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Immunization Therapies in the Prevention of Diabetes

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Insulin-dependent diabetes (IDD), being an autoimmune disease, offers several opportunities for immunological interventions that may result either in the reduction of disease severity or in delaying diabetes onset. Among the various experimental preventative approaches, parenteral immunization with islet-specific autoantigens appears to be practically simpler and promising. We have previously shown that immunization with insulin, insulin B chain and B chain epitope (p9–23), but not insulin A chain, in incomplete Freund's adjuvant (IFA) and in alum (with B chain) delayed/prevented diabetes onset in NOD mice. Here we demonstrate the protective efficacy of affinity purified GAD_{65} in IFA. While both insulin B chain and GAD_{65} significantly delayed the onset of diabetes ($P=0.001$), a recently described tyrosine phosphatase (IA–2) antigen did not ($P=0.38$). Interestingly, B chain immunization reduced the incidence of cyclophosphamide (CY)-accelerated diabetes by about 50–55%. We also provide further evidence that B chain, upon increased adsorption to alum, could improve on its protective capacity in NOD mice.

Introduction

Insulin-dependent diabetes (IDD) is a genetically influenced autoimmune disease caused by the progressive ablation of insulin-secreting pancreatic β-cells by autoreactive T lymphocytes. Targeted autoantigens include insulin and glutamic acid decarboxylase (GAD). Recently, a transmembrane protein, IA–2 (105 kDa), that belongs to the protein tyrosine phosphatase family, has been shown to be a major autoantigen in IDD patients [1]. The IA–2 gene seems to be expressed in human, mouse and rat insulinoma cells as well as enriched islets [2].

Parenteral insulin replacement has been the major treatment for over seven decades in the clinical management of IDD, although near-normal glucose levels by such means for prolonged periods is seldom achieved. This failure leads to diabetes associated complications. Recent advances in our understanding of the immunopathogenesis of IDD has led several investigators to propose potential immunological intervention therapies to prevent or delay IDD onset in subjects at risk of developing the disease. In recent years, several successful therapeutic studies have led to the development of NIH supported DPT–1 trial in humans.

The evolution of autoimmunity and insulitis prior to the onset of clinical diabetes in non-obese diabetic (NOD) mice, as in human diabetes, has enabled investigators to utilize this animal model to design effective immunotherapies. Based on such animal studies, it is now known that autoreactivity of IDD can be manipulated by various islet cell autoantigen-based procedures that may induce either downregulatory processes (e.g. clonal anergy and deletion of autoreactive T cells) or systemic deviation of autoimmune responses from destructive to non-destructive outcomes (e.g. Th2-dominated autoimmune responses, and transferable suppression). Intravenous (i.v.), oral and subcutaneous administrations of islet antigens have been reported to delay diabetes onset with or without reducing insulitis in NOD mice[3, 4]. In humans, oral antigen therapies using myelin basic protein have been tested in a pilot trial in multiple sclerosis patients [5] and another trial is currently being undertaken by our group in newly diagnosed diabetic patients. However, oral therapies require higher doses of antigens given in multiple feedings (in both mice and humans) in order to achieve significant effects [6–8], while the delaying effect of i.v. administrations in NOD mice is limited and depends on the dose and antigen (unpubl. obs.). Recently, intranasal administrations of insulin B chain epitope (p9–23) and GAD_{65} peptides have been reported to induce protection from diabetes and dominant Th2 responses [9, 10]. Our laboratory has demonstrated the protective effect of low dose subcutaneous insulin/insulin B chain immunizations in incomplete Freund's adjuvant (IFA) [4]. This protocol has also been successfully utilized by others using GAD_{67} in NOD mice [11]. In

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order to explore further the influence of subcutaneous immunizations in delaying diabetes onset in NOD mice, we have used GAD_{65} and IA–2 antigens in IFA. We have also investigated the effect of insulin B chain immunization on CY-accelerated diabetes. Further, the protective effect of B chain adsorbed to alum has been improved by prolonging the adsorption time.

Methods

Subcutaneous immunizations of NOD mice

Female NOD mice aged 3 weeks were purchased from Taconic Farms (German Town, NY) and housed in specific pathogen-free (SPF) conditions at the University of Florida Animal Resources Center. Control mouse strains such as Balb/c, CBA, and B6 were obtained and housed in SPF conditions at the animal resources center. Quantities of 100 µg of human recombinant insulin A or B chain (kindly provided by Dr Ron Chance, Eli Lilly, Indianapolis, IN), affinity-purified human or pig brain GAD_{65}, purified IA–2 protein (expressed as recombinant-GST fusion protein in *E. coli*, or glutathione-s-transferase (GST) fusion protein were administered. These agents were given subcutaneously in the inguinal and auxiliary regions in IFA, in alum (insulin B chain), at 4, 8 and 12 weeks of age. Equal volumes of IFA (GIBCO, Grand Island, NY) or 1:4 diluted Imject alum (Pierce, Rockford, IL) were used to emulsify/mix with insulin A/B chains. In alum studies, insulin B chain was adsorbed either for 30 min at RT or 18 h at 4°C following initial 30 min mixing as suggested by the manufacturer. Briefly, GAD was purified from fresh pig brain tissue and human recombinant GAD_{65} baculovirus-infected insect cell lysates (Syva Company, Palo Alto, CA) using GAD–1 monoclonal antibody-coupled CNBR-activated sepharose 4B (Pharmacia, Uppsala, Sweden) affinity column as described previously [12]. The pig brain lysates, GAD-depleted by repeated passage through affinity column (pExt), and insect cell lysates (vecLys) were used in IFA as controls. In CY studies, 5-week-old female NOD mice received in total two intraperitoneal (i.p.) injections of CY (Sigma Chemical Co, St Louis, MO), 2 weeks apart, (300 mg per injection for each kg of body weight). Only two immunizations (at 4 and 8 weeks of age) of B chain in IFA were made in CY experiments. Blood glucose levels were determined with Chemstrip bG (Boehringer Mannheim, Indianapolis, IN) and diabetes was diagnosed when hyperglycemia of >240 mg/dl was found in 2 consecutive weeks. In CY studies, 5 weeks after the last CY injection (i.e. at 12 weeks of age) blood glucose levels were determined.

Radioimmunoprecipitation of in vitro *translated IA–2 antigen*

Immunoprecipitation was carried out as previously described [2]. Briefly, the full-length human IA–2 cDNA without the leader sequence was cloned into a pCRII cloning vector (Invitrogen, San Diego, CA) with a perfect Kozak translational start sequence (GCGCCACCATGG). Plasmid DNA (1 µg) was added to TNT coupled rabbit erythrocyte lysate system (Promega, Madison, WI) in the presence of [^{35}S] (Amersham, Arlington Heights, IL) at 30°C for 2 h. Radiolabelled protein was determined by 10% trichloracetic acid precipitation. Immunoprecipitation was performed by mixing translated reticulocyte lysate and 5 µl of test serum in 100 µl of immunoprecipitation buffer. The reaction mixture was incubated at 4°C overnight, and 50 µl of 50% (vol/vol) protein A-agarose was added to the solution at 4°C for 1 h. The immunoprecipitation mixture was washed four times with immunoprecipitation buffer, boiled in sample buffer, and applied to a 8% SDS-PAGE gel. The gels were fixed and then exposed to film overnight. Serum that precipitated a 106 kDa band was considered to be positive.

Measurement of antibodies to insulin

To measure insulin-specific antibodies, 96-well plates were coated with 10 µg/ml of recombinant crystalline human insulin (Boehringer Mannheim, Indianapolis, IN) at 4°C overnight and blocked at room temperature with 5% BSA in PBS for 2 h. Sera from 13-week-old immunized mice (n=3–5) were used at a 1:100 dilution to detect the antibodies. HRP-conjugated second antibodies to mouse IgG isotypes were used as suggested by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Plates were developed with TMB/peroxidase substrate and peroxidase solution B (KP Laboratories, Gaithersburg, MD). The color reaction was stopped by adding 1N sulfuric acid, and plates were read at 450 nm using a Syva MicroTrak EIA Autoreader (Syva Company, Palo Alto, CA).

Statistics

The method of Kaplan and Meier [13] was used to construct life tables, and logrank chi-square statistics were used to compare them [14]. A Student's t-test or one-way ANOVA was used to compare the means. P values were calculated for two-sided comparisons. When multiple comparisons were made, the Bonferroni correction was applied.

Results

Onset of diabetes delayed in NOD mice immunized with insulin and GAD_{65}, but not with IA-2 antigen

Subcutaneous immunizations with insulin B chain and affinity purified pig brain GAD_{65}, or human recombinant insect cell-expressed GAD_{65}, significantly delayed the onset of diabetes in NOD mice

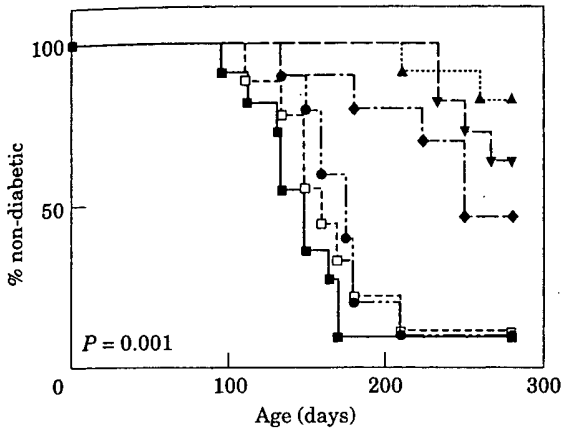

Figure 1. Delayed onset of diabetes in mice immunized with insulin B chain and GAD_{65} antigens in IFA. The survival curve shows the probability of remaining non-diabetic among various treated groups of mice. Female NOD mice subcutaneously immunized (as described in Methods) with insulin B chain (n=12), GAD_{65} (from pig brain (n=15) or human recombinant GAD_{65} (n=15)) in IFA showed a significant delay in the onset of diabetes (P=0.001) while PBS or GAD-depleted lysates did not (n=10–15). —■—: PBS; --▲--: B chain; --▼--: $pGAD_{65}$; --◆--: $hGAD_{65}$; --●--: vecLys; --□--: pExt.

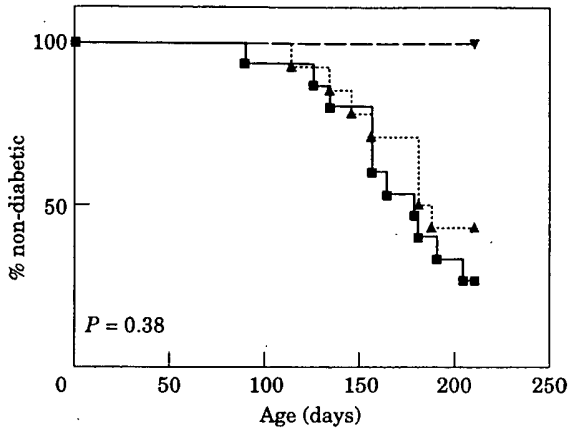

Figure 2. Immunization with IA–2 tyrosine phosphatase antigen did not confer protection. Female NOD mice were subcutaneously immunized in IFA with either IA–2 antigen (n=14) or GST fusion protein (n=15). B chain-immunized mice were kept as positive controls (n=8). There was no induction of protection by IA–2 (P=0.38). —■—: GST; --▲--: IA–2; --▼--: B chain.

(P=0.001). Control groups that received PBS or GAD-depleted (by repeated passage through GAD–1 affinity column) insect cell lysate (vecLys) or GAD-depleted pig brain extract (pExt) in IFA did not experience any protection (Figure 1). Thus, this observation extends the usefulness of IFA immunization protocol to GAD_{65}. The diabetic incidence at the end of the study was 17% for B chain-immunized mice and 36 and 50% for pig brain GAD and human GAD_{65}, respectively. Although there was a lower diabetic incidence in B chain immunized mice, the overall survival curve comparisons did not show significant differences among these groups (P=0.16). Unlike the effects of B chain and GAD_{65}, there was no beneficial protective effect with IA–2 antigen immunization in NOD mice compared to control mice immunized with GST fusion protein (P=0.38) (Figure 2). Data in Table 1 show that there were no spontaneous autoantibodies to IA–2 antigen in the sera from 13-week-old NOD mice. However, immunization with IA–2 resulted in the production of IA–2 antibodies. Sera from NOD mice of various ages have been found to lack spontaneous antibodies to IA–2 (M. Lan, unpubl. obs.).

When subcutaneously immunized with alum+B chain (B chain adsorbed to alum for 30 min at RT), a significant level of protection was observed compared to untreated and alum+A immunized mice (P=0.012) (Figure 3A). A non-specific delaying effect was seen with alum alone, but that effect did not reach statistical significance at the end of >300 days of study (P=0.22). In this study we show that the protection offered by alum+B could be further improved by increasing the time of adsorption to 18 h following the initial 30 min mixing (Figure 3B) (P=0.0007). This also reduced non-specific effects of alum considerably.

Table 1. *Sera from IA–2 immunized NOD mice precipitated radiolabelled IA–2*

Sera	Anti-IA–2
NOD (n=3)	Negative
NOD-imm with GST (n=5)	Negative
NOD-imm with IA–2 (n=5)	**Positive**
CBA (n=3	Negative

Sera from 13-week-old female NOD mice that were either IA–2 or GST immunized, and control mice, were analysed for IA–2 reactivity by immunoprecipitation assay. The immunoprecipitation mixture was boiled in sample buffer before running on a 8% SDS-PAGE gel. After overnight exposure to film, the sera that exhibited the 106 kDa band were scored positive.

To assess further the degree of IDD resistance conferred by insulin B chain immunization in IFA, the immunization schedule was coupled with i.p. injections of CY. As shown in Table 2, among B chain-immunized mice, 50–55% remained free of diabetes compared to mice immunized with IFA alone (P<0.05). This finding clearly demonstrates the 'strength' of protective mechanisms induced by the B chain immunization procedure.

Induction of anti-insulin antibodies in non-susceptible mouse strains

While NOD mice benefit from autoantigen immunization therapies, it is essential to investigate the effect of such therapeutic procedures in diabetes non-susceptible mouse strains. Our results in Figure 4 demonstrate the induction of insulin-specific anti-

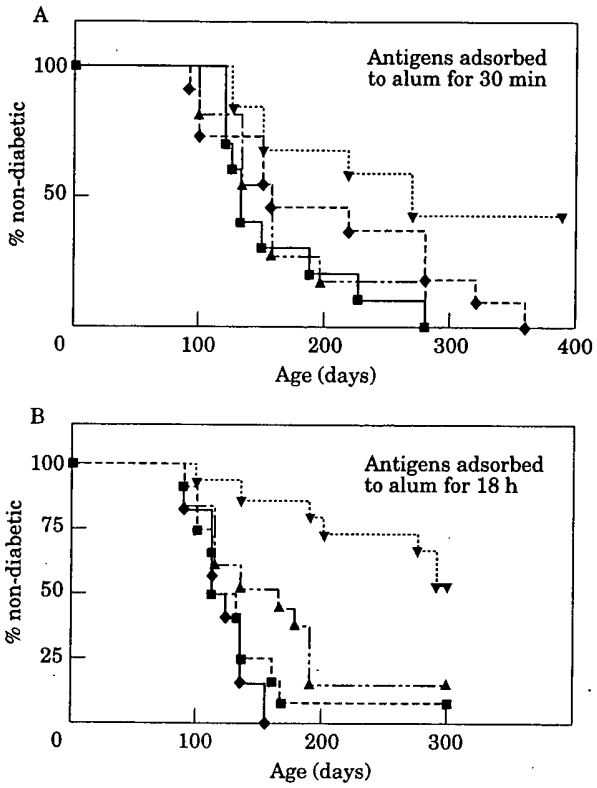

Figure 3. Immunization with insulin B chain adsorbed to alum protected NOD mice from diabetes. Female NOD mice were subcutaneously immunized with B chain adsorbed to alum for 30 min at RT, or adsorbed for 18 h at 4°C (as described in Methods). Only B chain (A, $P=0.011$; B, $P=0.0007$), but not A chain in alum led to a considerable delay in the onset of diabetes. The prolonged adsorption of B chain to alum seemed to improve the observed protection, as suggested by P-values. – –◆– –: Alum; – –▲– –: alum+A; – –▼– –: alum+B; —■—: untreated.

Table 2. *Resistance to cyclophosphamide-accelerated IDD in insulin B chain immunized NOD mice*

Treatment groups	Diabetic incidence (%)
Untreated	0
IFA+CY alone	75
B chain immunization in IFA+CY	30

Four-week-old female NOD mice were injected twice with CY (300 mg/kg wt i.p., 14 days apart). Five weeks after last CY injection, incidence of diabetes was determined by measuring plasma glucose levels. Glucose values >240 mg/dl were considered diabetic. The incidence of CY-accelerated IDD was less than that of the sex- and age-matched control group ($P=0.05$).

bodies (of IgG1, 2a and 2b isotypes) upon immunization with insulin in Balb/c, B6 and CBA strains, as tested with sera from 13-week-old mice. The level of antibodies in the preimmune IDD non-susceptible strains reaches that of background O.D. values (data not shown). Unlike NOD mice (which express spontaneous insulin and GAD-specific antibody

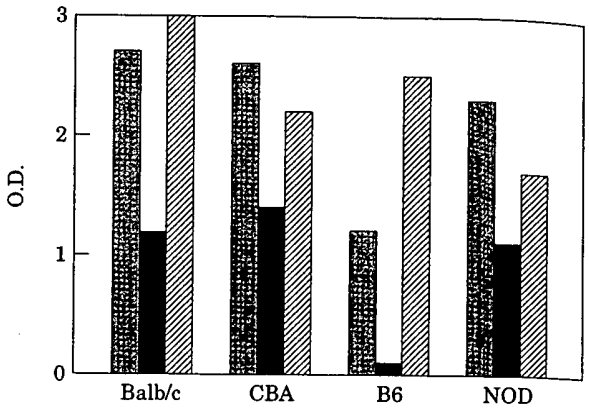

Figure 4. Induction of antibody responses to insulin in B chain-immunized non-susceptible mouse strains. Female Balb/c, CBA, B6, and NOD mice were immunized with insulin B chain in IFA as described in Methods. Serum samples were obtained at 13 weeks of age and were tested for insulin-specific antibodies by ELISA. A representative set of results are expressed in O.D. values. The level of pre-immune insulin-specific antibodies of IDD non-susceptible strains reached that of background O.D. values (data not shown). While there were no spontaneous anti-insulin antibodies in diabetes non-susceptible strains, immunization with insulin B chain resulted in the production of insulin antibodies. ▨: IgG1; ■: IgG2a; ▤: IgG2b.

responses of IgG2b isotype [15]), none of these strains expressed spontaneous insulin antibodies. These mice did not express insulitis, or have diabetic symptoms (data not shown). Thus, in non-susceptible strains, although humoral response to insulin is induced, it is of no clinical significance.

Discussion

As the understanding of the autoantigens involved in the pathogenesis of IDD increases, the possibility of using these autoantigens in antigen-specific immunotherapies for IDD intervention becomes increasingly feasible. We have previously demonstrated the potential of autoantibodies to GAD_{65}, insulin and islet cell cytoplasmic antigens (ICA) in the prediction of IDD development in human subjects at risk of the disease [16]. Associations between low GAD_{65} autoantibody levels and high T cell proliferations to the same antigen, and between low T cell proliferations and high autoantibody levels to GAD have been found to give differential risks [17], suggesting the importance of GAD autoimmunity to IDD. Although the predictive power of insulin autoantibody for impending IDD by itself is relatively low, there is little information yet on insulin-specific T cells in IDD pathogenesis. Previously, autoimmune responses to insulin had not been considered to be important in the induction of IDD [18]. However, in the pancreatic islets of NOD mice, a higher frequency of insulin-specific T cells has been found [19]. This suggests a pathogenic role for insulin-specific responses in IDD either as disease-promoting effector cells or as

protective regulatory elements. Recently, IA–2 tyrosine phosphatase protein has been shown to react with 66% of diabetes patients. Greater than 90% of ICA+ve but GAD_{65} antibody −ve sera had antibodies to IA–2. Further, IA–2β (another tyrosine phosphatase antigen) and IA–2 have been demonstrated to be the precursors for 37 and 40 kDa islet cell antigens, respectively [1, 20]. For these reasons we used insulin B chain, GAD_{65}, and IA–2 antigens to analyse their protective efficacies in the immunization therapies aimed at preventing IDD.

The present study confirms the protective effects of insulin B chain, while documenting the protective effects of GAD_{65} immunizations in IFA (Figure 1). Previously, Elliot *et al.* [11] demonstrated the benefits induced by GAD_{67} immunization therapy in NOD mice. Surprisingly, such a protective effect could not be shown with IA–2 antigen (Figure 2). Unlike insulin and GAD_{65} autoantibodies, it has not yet been possible to detect spontaneous autoantibodies to IA–2 antigen in NOD mice (Table 1). However, there is no intrinsic self-tolerance to IA–2, as demonstrated by the presence of specific antibodies in the immunized mice. It is not clear why there are no detectable spontaneous antibodies to IA–2 in NOD mice, or whether the absence of spontaneous response is in any way related to the inability of IA–2 antigen to protect NOD mice from diabetes. This may reflect the list of subtle differences that seem to exist between human and mouse diabetes. It is encouraging that alum, which is widely used in humans as adjuvant, seems to improve the protection offered by B chain upon increased adsorption time (Figure 3). It is not known whether increasing the adsorption time correspondingly increases the amount of B chain adsorbed.

Cyclophosphamide is known to accelerate diabetes in NOD mice [21] and has been shown to be associated with increased expression of inducible nitric oxide synthase (iNOS) in macrophages and enhanced production of IFN-γ by Th1 cells [22]. Immunosuppressants such as sodium fusidate and immunomodulating (non-depleting) anti-CD4 antibody have been demonstrated to reduce the incidence of CY-accelerated diabetes in NOD mice [23]. The current observation suggests such considerably CY resistant protection with insulin B chain immunizations. However, the level of protection remained between 55 and 60% compared to the CY-treated control group (Table 2). Perhaps these aforementioned beneficial treatments may reduce inflammatory Th1 responses, while augmenting or maintaining Th2 responses.

Taken together, it is clear that immunization therapies are helpful in delaying IDD onset in NOD mice. However, since the ultimate goal has been prevention of human diabetes through potentially useful vaccination/immunization therapies, one has to investigate, for reasons of safety, the immunological consequences of such therapies in people who may not be at risk of developing diabetes. Towards this end, we have used insulin B chain immunizations in diabetes non-susceptible mouse strains such as Balb/c, CBA, and B6. Our preliminary data suggest that these strains could mount effective humoral responses to insulin upon immunizations (Figure 4), without expressing any other harmful clinical consequences such as insulitis and hyperglycemia (data not shown). However, a long-term study of this kind, perhaps with GAD_{65} antigen as well, should be conducted in the future. Such studies should involve measuring not only immune parameters, but also the impact of prolonged islet antigen-specific antibody presence on endogenous insulin secretion by pancreatic β-cells, and insulin resistance if any.

As to mechanisms of protection in B chain immunized mice, we have previously demonstrated a significant reduction in IFN-γ mRNA within the islet infiltrates [4]. Using DTP as adjuvant, increased Th2 cytokine mRNA levels have been reported to be associated with protection [24]. Based on our observations and those of others, we speculate that when some 'powerful' immune responses are intentionally induced (as in immunization therapies) during the initiation of spontaneous immune responses in NOD mice, these intentionally induced responses may influence the course of spontaneous autoimmune responses. However, this may not be feasible if a beneficial response is induced at a later time point when full-blown autoreactivities have already been established. Finally, the information on the temporal relationship between intentional immunization and the initiation of autoimmune reactivities to islet antigens is very essential, as described above, and is yet to be studied in detail. These data will eventually provide insights of relevance to future human trials of diabetes prevention.

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